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OBSTETRICS

Successful use of an artificial placenta—based life support system to treat extremely preterm ovine fetuses compromised by intrauterine inflammation

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BACKGROUND: Ex vivo uterine environment therapy is an experimental intensive care strategy for extremely preterm infants born between 21 and 24 weeks of gestation. Gas exchange is performed by membranous oxygenators connected by catheters to the umbilical vessels. The fetus is submerged in a bath of synthetic amniotic fluid. The lungs remain fluid filled, and pulmonary respiration does not occur. Intrauterine inflammation is strongly associated with extremely preterm birth and fetal injury. At present, there are no data that we are aware of to show that artificial placenta—based systems can be used to support extremely preterm fetuses compromised by exposure to intrauterine inflammation.

OBJECTIVE: To evaluate the ability of our ex vivo uterine environment therapy platform to support extremely preterm ovine fetuses (95-day gestational age; approximately equivalent to 24 weeks of human gestation) exposed to intrauterine inflammation for a period of 120 hours, the following primary endpoints were chosen: (1) maintenance of key physiological variables within normal ranges, (2) absence of infection and inflammation, (3) absence of brain injury, and (4) gross fetal growth and cardiovascular function matching that of age-matched in utero controls.

STUDY DESIGN: Ten ewes with singleton pregnancies were each given a single intraamniotic injection of 10-mg *Escherichia coli* lipopolysaccharides under ultrasound guidance 48 hours before undergoing surgical delivery for adaptation to ex vivo uterine environment therapy at 95-day gestation (term=150 days). Fetuses were adapted to ex vivo uterine environment therapy and maintained for 120 hours with constant monitoring of key vital parameters (ex vivo uterine environment group) before being killed at 100-day equivalent gestational age. Umbilical artery blood samples were regularly collected to assess blood gas data, differential counts, biochemical parameters, inflammatory markers, and microbial load to exclude infection. Ultrasound was conducted at 48 hours after intraamniotic lipopolysaccharides (before surgery) to confirm fetal viability and at the conclusion of the experiments (before euthanasia) to evaluate cardiac function. Brain injury was evaluated by gross anatomic and histopathologic investigations. Eight singleton pregnant control animals were similarly exposed to intraamniotic lipopolysaccharides at 93-day gestation and were killed at 100-day gestation to allow comparative postmortem analyses (control group). Biobanked samples from age-matched saline-treated animals served as an additional comparison group. Successful instillation of lipopolysaccharides into the amniotic fluid exposure was confirmed by amniotic fluid analysis at the time of administration and by analyzing cytokine levels in fetal plasma and amniotic fluid. Data were tested for mean differences using analysis of variance.

RESULTS: Six of 8 lipopolysaccharide control group (75%) and 8 of 10 ex vivo uterine environment group fetuses (80%) successfully completed their protocols. Six of 8 ex vivo uterine environment group fetuses required dexamethasone phosphate treatment to manage profound refractory hypotension. Weight and crown-rump length were reduced in ex vivo uterine environment group fetuses at euthanasia than those in lipopolysaccharide control group fetuses ($P<.05$). There were no biologically significant differences in cardiac ultrasound measurement, differential leukocyte counts ($P>.05$), plasma tumor necrosis factor α, monocyte chemoattractant protein-1 concentrations ($P>.05$), or liver function tests between groups. Daily blood cultures were negative for aerobic and anaerobic growth in all ex vivo uterine environment group animals. No cases of intraventricular hemorrhage were observed. White matter injury was identified in 3 of 6 lipopolysaccharide control group fetuses and 3 of 8 vivo uterine environment group fetuses.

CONCLUSION: We report the use of an artificial placenta—based system to support extremely preterm lambs compromised by exposure to intrauterine inflammation. Our data highlight key challenges (refractory hypotension, growth restriction, and white matter injury) to be overcome in the development and use of artificial placenta technology for extremely preterm infants. As such challenges seem largely absent from studies based on healthy pregnancies, additional experiments of this nature using clinically relevant model systems are essential for further development of this technology and its eventual clinical application.

Key words: artificial placenta, chorioamnionitis, extremely preterm infants, ex vivo uterine environment therapy, fetal inflammatory response, fetal brain injury

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Preterm birth (delivery before 37 weeks' gestation) is a leading cause of death among children younger than 5 years and of long-term disability.[1] Globally, more than 1,000,000 premature babies die each year.[2] Overall outcomes for preterm infants have markedly improved over the past 5 decades as a result of advances in neonatal and obstetrical care including antenatal steroid therapy and postnatal administration of exogenous surfactant.[3]

Infants born at the border of viability (21—24 weeks' gestation) continue to have an extremely high risk of death or severe complications.[1] Outcomes for extremely premature (<28 weeks' gestation) and extremely low

AJOG at a Glance

Why was this study conducted?

To determine the ability of a refined artificial placenta—based life support platform to maintain extremely preterm ovine fetuses exposed to intrauterine inflammation, a common antecedent of extremely preterm birth.

Key findings

Our artificial placenta platform was successfully used to maintain 8 extremely preterm fetuses for a predetermined period of 120 hours. Biological parameters and cardiovascular performance were largely equivalent to in utero controls. Infection or additional injury was not identified.

What does this add to what is known?

This study presents the first data, of which we are aware, demonstrating the ability of an artificial placenta—based life support platform to support extremely preterm ovine fetuses compromised by exposure to intrauterine inflammation. Intrauterine inflammation is a common antecedent of extremely preterm birth and is associated with adverse outcomes. These data underscore the potential clinical application and highlight the additional refinements required before an artificial placenta—based system can be considered a viable treatment option for extremely preterm infants born at the border of viability.

birthweight infants (ELBW; <1000 g) have improved little over the past 2 decades.[1,4–6] One of the factors responsible for this relative lack of improvement in outcomes is that ventilation-based life support systems may have reached an efficacy threshold when used to support the extremely underdeveloped cardiopulmonary system.[6,7]

We have developed an experimental treatment platform for extremely preterm infants, that is, ex vivo uterine environment (EVE) therapy.[8–11] The central principle underlying the iterative development of this platform is to treat extremely preterm infants as fetuses, rather than as small babies, and in doing so avoid the use of pulmonary gas exchange. Although longer survival with a range of comparatively mature fetal models has been repeatedly demonstrated in sheep and goats over the past 30 years (broadly corresponding to 28—32 weeks' gestation in humans),[9,12–15] the feasibility of using an artificial placenta—based system to successfully maintain extremely preterm fetuses representative of the developmental stage of extremely preterm human fetuses was only recently demonstrated for the first time.[8]

One significant question that has not been addressed over the 60-year history of artificial placenta development is whether or not such a system could be used to support compromised fetuses.[8–15] This is an extremely important consideration given that intrauterine infection and inflammation are common antecedents of extremely preterm labor (PTL) and likely affect the ability of a fetus to adapt to artificial placenta—based treatments. It is reported that 40%—70% of cases of PTL are associated with chorioamnionitis, with prevalence correlated to fetal gestational age (GA) at preterm delivery.[16,17] For example, chorioamnionitis is observed in nearly 70% of deliveries at 24 weeks' gestation or earlier, but in only 16% of cases delivered at 34 weeks' gestation.[18]

The presence of a fetal inflammatory response is strongly associated with not only PTLs but also a higher rate of fetal complications and death.[19,20] In particular, inflammation in response to microorganisms in the amniotic environment is associated with life-threatening lung injuries represented by bronchopulmonary dysplasia[21,22] and neonatal brain injuries represented by white matter injury (WMI) or hemorrhage, resulting in the subsequent diagnosis of cerebral palsy.[23,24] We suggest that for artificial placenta—based systems to be used clinically, the technology must be capable of supporting extremely preterm fetuses compromised by exposure to intrauterine inflammation.

We report the ability of our EVE therapy platform to support extremely preterm lambs (95-day GA; weighing approximately 600 g; equivalent to approximately 24 weeks' GA in humans) after exposure to intrauterine inflammation caused by intraamniotic (IA) exposure to *Escherichia coli* lipopolysaccharides (LPS)[25,26] for a period of 120 hours. Although LPS elicits a sterile inflammatory response (as distinct from the use of viable microorganism), its use has the advantage of allowing the generation of a well-characterized and strong intrauterine inflammatory response, chorioamnionitis, and pathologic changes in a number of fetal systems including the lung and brain.

A 120-hour maintenance period was chosen for this study based on clinical data showing that the preponderance of death and injury identified in extremely preterm infants occurs acutely after delivery. In addition, in our experience, adverse outcomes in the EVE therapy model similarly occur within 2—3 days of establishment of the life support platform. The primary outcome measures were (1) maintenance of key physiological parameters, (2) absence of infection and inflammation, (3) absence of brain injury, and (4) growth and cardiovascular function patterns matching those of noninstrumented, age-matched in utero controls.

Materials and Methods

Experimental protocol

All procedures and measurements were performed in Perth, Western Australia, after review and approval by the Animal Ethics Committee of the University of Western Australia (RA/3/100/1378). This study consisted of 2 experimental groups:

1. An IA LPS-exposed EVE therapy group (EVE group) comprising fetuses exposed to LPS for 2 days before delivery and maintenance on EVE therapy for 5 days (n=10)

2. An IA LPS control group (LPS control group) comprising fetuses remaining in utero for 7 days after LPS exposure (n=8)

Additional tissues from age-matched, saline-exposed animals were obtained from our BioBank[8,26] to establish a third saline control group (3) to allow for an LPS-naive comparison with LPS-exposed tissues.

Study protocol

Ex vivo uterine environment group

Ten Merino cross ewes (term: approximately 150 days) with timed, singleton pregnancies were given single 10-mg IA LPS injection under ultrasound guidance at a GA of 92−93 days with successful amniotic fluid (AF) placement confirmed, as described previously.[25,26] Fetuses were surgically delivered 48 hours later at 94- to 95-day GA and adapted to EVE therapy as follows.

Surgical delivery. Ewes were fasted for 12 hours before surgery with ad libitum access to water. Ewes were premedicated, anesthetized, intubated, and ventilated (acepromazine 0.03 mg/kg and buprenorphine 0.01 mg/kg intramuscularly, midazolam 0.25 mg/kg and ketamine 5 mg/kg intravenously, 1%−2% isoflurane in 100% oxygen inhaled, tidal volume 10 mL/kg 8−10 breaths/min) during the surgical procedure. Intravenous fluids (0.9% NaCl) were administered at a rate of 10 mL/kg/h. The ewe's abdomen was clipped to expose the skin and thoroughly prepared for surgery, as described previously.[8−11,27] Ewes in both the EVE and control groups were killed with an intravenous bolus of pentobarbitone (160 mg/kg). After a maternal laparotomy and hysterotomy, the fetuses were placed inside the sterilized artificial uterus, with care taken to ensure umbilical cord patency. Fetuses were intermittently bathed with sterile saline warmed to 40°C. The catheterization procedure was performed before delivery as follows:

1. In a procedure lasting approximately 10−15 min, 1 umbilical artery was catheterized (8 Fr, Bio-Medicus 1-piece femoral venous cannulae; Medtronic, Minneapolis, MN) and secured approximately 7−8 cm outside the umbilical ring. The tip of the arterial catheter was sited approximately 2−3 cm from the umbilical ring.
2. One umbilical vein was then quickly cannulated with an 8-Fr custom-made catheter (Nipro Corporation, Osaka, Japan) and secured approximately 1.5−2 cm external to the umbilical ring. The tip of the venous catheter was sited approximately 0.5−1 cm past the umbilical ring. The fetuses were then attached to 1 membranous oxygenator.
3. A second umbilical artery was catheterized while the circuit was temporally circulated with 1 umbilical artery and umbilical vein and then connected to the circuit.
4. Finally, the fetus was carefully transferred to the site to be maintained, and the bag was promptly filled with synthetic AF.

Maintenance after delivery

EVE group fetuses were maintained and observed in parallel by a single investigator on a rotating 24-hour shift; 24 hours after commencement of the EVE therapy, normal intermittent active fetal swallowing movements, breathing movements, gross fetal body movements, and flexure and extension of limbs were assessed at least every 6 hours. The presence of edema, ascites, pleural effusions, or bleeding was determined by ultrasound (single operator) and by gross examination during necropsy after 120 hours of EVE therapy.

Fetuses were continuously treated with intravenous heparin (12.5 U/kg/h) to prevent blood coagulation, with the dose adjusted after stabilization in an attempt to maintain an activated clotting time of 180−250 seconds. Prostaglandin E_1 (PGE_1) (40 ng/kg/min, Tandetron; Takata Pharmaceutical, Saitama, Japan) was continuously administered after delivery. Phosphodiesterase type 3 inhibitor (0.5 μg/kg/min, Primacor; Sanofi, Sydney, Australia) was given to assist maintenance of organ blood flow.[28] Midazolam was continuously administered for the first 6 hours (0.15 mg/kg/h).[8] Hypoxia is characteristic for fetuses exposed to chorioamnionitis and a significant factor for severe brain injury. Oxygen supply to the membranous oxygenators was strictly adjusted to maintain fetal PaO_2 within a normal range between 15 and 30 mm Hg.[29−31] The fetuses were maintained with EVE therapy for 120 hours, followed by euthanasia with intravenous pentobarbitone (160 mg/kg/dose) for measurements of body weight (BW), crown-rump length (CRL), and tissue sample collection (lung, brain).

Management of refractory hypotension

We defined refractory hypotension as hypotension resulting in a decrease of circuit flow below 150 mL/kg/min (normal circuit flow is 150−250 mL/kg/min),[32−34] which required glucocorticoid administration in addition to volume expansion (10−20 mL/kg/h) with fresh-frozen plasma (FFP) to be resolved. To prevent critical refractory hypotension (which is frequently observed in extremely preterm infants at the border of viability), intravenous hydrocortisone (HC) (3 mg) was administered to all fetuses immediately after EVE therapy induction, followed by 6-hourly administrations at a dose of 3 mg (estimated 3−4 mg/kg, approximately 60 hours) and 2 mg (estimated 2−3 mg/kg, approximately 120 hours).[8] If refractory hypotension could not be controlled after primary administration of HC, a second dose of HC (3 mg) and increased volume expansion (30 mL/kg/h) and finally intravenous dexamethasone (Dex; 0.15mg/dose, estimated 0.25 mg/kg/dose; Hospira Australia, Melbourne, Australia) was provided. Hypotension and circuit flow were evaluated every 5−10 min after HC or Dex injection. Iterative Dex was provided until hypotension and circuit flow were managed within normal range. FFP was provided for volume expansion according to hypotension for the first 4−6 hours (10−30 mL/kg/h) to all fetuses.[8] Inotropic drug use was avoided to prevent contraction of the umbilical arteries.

Parental nutrition

Intravenous nutrition comprised glucose (9.5%−10%), amino acids (3−3.2 g/kg/d, Pleamin-P injection; Fuso Pharmaceutical, Osaka, Japan), lipid (0.1 g/kg/d, Intralipid 20%; Fresenius Kabi Australia, Sydney, Australia), vitamin compounds (1/8 vial/d, Daimedin multi injection; Nichi-Iko Pharmaceutical, Tokyo, Japan), micronutrient (0.1 mL/d, Cizanarine N injection; Nissin Pharmaceutical, Yamagata, Japan) was administered to provide 70−75 kcal/kg/d.[8]

Prevention of infection

Bacterial infection was determined based on a positive finding of bacteremia by microbial cultures. Meropenem (15 mg/kg/dose, Ranbaxy; Sydney, Australia) was administered intravenously to the fetuses every 6 hours. Intravenous fluconazole (4 mg/kg/dose, Fluconazole-Claris; AFT Pharmaceuticals Pty Ltd, Sydney, Australia) was administered to the fetuses every 24 hours.[8]

Collection of maternal blood for fetal transfusion

Meropenem (1 g/dose) was administered to each ewe after induction of anesthesia; 100 mL (approximately 2%−3% of total circulating blood volume for ewes) was aseptically collected from the jugular vein before surgery. Whole blood was immediately heparinized and then used for priming of the artificial placenta circuit. A further 300−400 mL of whole blood was collected after fetal delivery using a triple-bag blood transfusion system (T331150, Fresenius Kabi, Mount Kuring-Gai, Australia). Packed red cells were preserved at 4°C before use. Fresh plasma was frozen at −80°C and defrosted and warmed to physiological temperature on demand.

Ex vivo uterine environment therapy system components

Artificial placenta. The circuit was composed of 3 main parts: (1) outflow tubes, (2) membranous oxygenators, and (3) an inflow tube (Nipro Corporation, Osaka, Japan). Only 1 membranous oxygenator per EVE fetus was used. Heparinized polyvinyl chloride tubes were used for both the inflow and outflow tubes. The circuit was primed with 50 mL of heparinized maternal blood. Lipo-PGE_1 was mixed in the circuit priming to prevent umbilical vessel and ductal contraction (100 ng/mL; Sawai Pharmaceutical, Osaka, Japan). The calculated membrane surface area for gas exchange was 0.15 m^2. Extracorporeal pumps were not used to maintain the circuit flow.

Amniotic fluid. Synthetic AF was aseptically prepared as follows: pH, 7.34±0.15; Na^+, 110±4 mEq/L; Cl^-, 111±4 mEq/L; K^+, 6.5±0.8 mEq/L; Ca^{2+}, 1.4±0.3 mEq/L; meropenem, 167 mg/L; fluconazole, and 3.3 mg/L (all values represent group mean±standard deviation [SD]). AF was preheated to 39.5°C−40.0°C and UV treated for a minimum of 6 hours before addition to the AF bath. The AF bath was filled with synthetic AF (6 L) and warmed constantly by 2 heaters. Heaters were installed at the top (radiant warmer) and at the bottom (contact heat pad) of the AF bath. After the fetus was submerged, AF was maintained at a constant temperature of 38.7°C±0.3°C (group mean±SD). To minimize the risk of microbial colonization, a transparent, sterilized plastic bag was used to contain the AF bath. The AF bath was rinsed and replaced every 6 hours after the start of EVE therapy with 30 L of new synthetic AF.

Physiological, hematological, biochemical, and microbiological data acquisition

Fetal heart rate (HR) and mean arterial pressure were continuously monitored and recorded using a SurgiVet monitor (Smiths Medical, St. Paul, MN). Circuit blood flow (mL/min) was continuously monitored using electromagnetic flow sensors (Transonic 400-Series, Transonic Systems Inc, Ithaca, NY) attached to the arterial positions of the blood circuit and recorded using a PowerLab (ADInstruments, Dunedin, New Zealand). Fetal umbilical arterial blood gases (pH, base excess [BE]), pCO_2, pO_2, O_2 saturation (SO_2), O_2 content (CtO_2), sodium ion (Na^+), potassium ion (K^+), calcium ion (Ca^{2+}), and chloride ion (Cl^-]); hemoglobin, lactate, and glucose level (Siemens RAPIDPoint 500, Munich, Germany); and activated clotting times (Hemochron Jr, Accriva Diagnostics, San Diego, CA) were measured at least every 6 hours. AF was collected at surgical delivery. Fetal umbilical arterial blood samples were collected every 24 hours after induction of EVE therapy.

Hematological analyses (white blood cell [WBC] counts and differential leukocyte counts), biochemical parameters (aspartate aminotransferase [AST], alanine aminotransferase [ALT], gamma-glutamyl transpeptidase [GGTP], glutamate dehydrogenase [GLDH], blood urea nitrogen [BUN], total bilirubin, albumin, magnesium [Mg], phosphorus [P], and BUN to creatinine ratio), endocrine parameters (cortisol, adrenocorticotropic hormone [ACTH], and insulin-like growth factor 1 [IGF-1]), and microbiological analysis (anaerobic and aerobic cultures) were performed by an independent clinical pathology laboratory (Vetpath, Perth, Australia). To prevent hypoxia due to anemia, all sampling was made volume neutral through addition of packed red blood cells and FFP. Packed red blood cell transfusion generated with maternal blood was performed (10 mL/kg/time) when hemoglobin values fell below 10 g/dL.

Ultrasound assessment of cardiac function

Ultrasound assessments were performed by a single operator to confirm normal fetal cardiac function at 48 hours after LPS injection (before surgeries for EVE animals) and to assess fetal cardiac function before euthanasia. Measurements were conducted with a Philips CX50 system, S5-1 phased array probe (Philips Healthcare, the Netherlands) and associated obstetrics software. For control animal measurements, ewes were held in a dorsal recumbency and the fetal position from the ventral aspect was confirmed. The ultrasound beam was focused to obtain a basal 4-chamber view, 5-chamber view, left ventricular outflow tract view, right ventricular

outflow tract view, or 3 vessel (3V) view to check the following items.[8,27]

Briefly, transtricuspid and transmitral inflow were measured using Doppler echocardiography to assess the peak early filling (E wave) and late diastolic filling (A wave) velocities in calculation of each E:A ratio. Cardiac time intervals such as isovolumetric contraction time (ICT), ejection time (ET), and isovolumetric relaxation time (IRT) were measured for the right and left ventricles. Myocardial Performance Index (MPI) was calculated using the formula MPI=ICT+IRT/ET.

Pulsed Doppler tracings were obtained at the point of the inferior vena cava orifice entering the right atrium. Peak velocity during atrial contraction (A), which frequently has reversed blood velocities away from the heart, and peak velocity during ventricular systole (S) were measured from the recorded flow velocity waveform, and the A:S ratio was calculated to obtain the preload index (PLI).

The inner diameter (D) of the aorta and the pulmonary artery was measured at an insonation angle perpendicular to the vessel wall, between the open semilunar valves. The systolic velocity time integral (TVI) and HR were calculated as an average of 4−6 cardiac cycles. Left and right ventricular output were calculated as $\pi \cdot (D/2)2 \cdot TVI \cdot HR$. The cardiac output (CO) was calculated as the sum of the two, and the corrected CO was calculated by dividing this by the fetal weight.[35]

Color flow Doppler imaging was used to detect the blood flow direction through the ductus arteriosus. Blood flow from the pulmonary artery to the descending aorta was determined as right to left directional flow. These measurements were performed before euthanasia.

Lipopolysaccharide control group

Eight Merino cross ewes with timed, and singleton pregnancies were given IA LPS injection (10 mg) at a GA of 92−93 days. After 7 days of ultrasound assessments, they were delivered and killed with an intravenous bolus of pentobarbitone (160 mg/kg) at a GA of 99−100 days to allow comparative measurement of BW, CRL, organ weights (lung, brain), tissue collection (lung for polymerase chain reaction [PCR], brain for histopathologic analysis), and fetal whole blood and plasma collection at delivery (immediately before euthanasia) to perform blood corpuscle counts, including differential leukocyte count and biochemical and cytokine analyses.

Saline control group (BioBank samples)

Comparative analysis of AF and plasma MCP-1 concentrations between LPS-exposed and naive fetal samples was performed using previously published, age-matched samples obtained from saline-exposed catheterized fetuses drawn from our ovine BioBank.[26] Saline control group blood gas data, WBC counts, differential leukocyte counts, plasma for cytokine, lung tissues for PCR, and brain tissues for histopathology presented herein were similarly obtained as a reference set from our published ovine BioBank.[8]

Laboratory analyses

Enzyme-linked immunosorbent assays

MCP-1 protein concentrations were measured using fetal plasma and AF samples from EVE group animals taken 48 hours after IA LPS injection.[26] Those values were compared with data from saline control and LPS control group animals. At the end of the experimental period, fetal plasma concentrations of MCP-1 and tumor necrosis factor alpha (TNF-α) were also compared between the EVE group animals and the saline and LPS control group animals.

Commercial kits from Kingfisher Biotech (St Paul, MN), with washing performed on a Biosan plate washer (Inteliwasher 3D-IW8, Biosan, Riga, Latvia), were used as previously described.[9] Standards (calibration curve, $R^2>0.99$) were assayed in triplicate (average coefficient of variation, 7.8%) and samples were assayed in duplicate. The assay limit of detection was <4 pg/mL; 100 μL of each standard or sample was incubated overnight (16 hours) at 4°C. Assays were performed according to the manufacturer's instructions, with absorbance at 450 nm read on a HiPo MPP-96 microplate photometer (Biosan, Riga, Latvia).

RNA extraction and quantitative polymerase chain reaction analysis of lung cytokine expression

Total RNA was extracted with TRIzol (Life Technologies) from 100 mg of snap-frozen fetal lung (right lower lobe).[26,36,37] Extracted RNA was treated with TURBO DNase (Life Technologies) according to the manufacturer's instructions. RNA template was quantified using a Qubit 2.0 fluorometer (Life Technologies) using a broad-range RNA quantitation kit (Life Technologies). RNA extracts were diluted in nuclease-free water (Life Technologies) to a final concentration of 25 ng/μL.

Ovine-specific PCR primers and hydrolysis probes for interleukin (IL)-1β, IL-6, TNF-α, and MCP-1 (Life Technologies) were used to perform quantitative PCR. Reactions were performed on a Viia7 thermocycler (Life Technologies) using an EXPRESS One-Step SuperScript qRT-PCR Kit (Life Technologies) with 125 ng of DNAse-treated template fetal tissue RNA in a total volume of 20 μL according to the manufacturer's instructions. Reaction cycling conditions were 15-minute reverse transcription at 50°C and an initial denaturation/polymerase activation at 95°C for 20 seconds, followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds (data acquisition phase). Target Cq values were normalized to 18S rRNA Cq value and expressed as fold changes relative to pooled control values. Reaction efficiencies were within limits proposed in the MIQE guidelines.[38] dCq values were used to perform statistical analyses for significant differences between intervention groups vs control group.

Preterm brain histology

Brain perfusions fixed with formalin were processed for gross anatomic investigation.[8] The left hemisphere was sectioned at 5-mm intervals along the coronal plane from the anterior to

posterior surfaces according to the method by Banker and Larroche.[39] Briefly, each 5-mm section was visually inspected for the presence of abnormalities (petechial hemorrhage, focal coagulation necrosis). Every second 5-mm section (4 in total) was selected to be embedded in paraffin; 10-μm sections were then hematoxylin and eosin (H&E) stained. Sections were evaluated for the presence of pathologic lesions by a single investigator. WMI was defined as the presence of focal coagulation necrosis and cellular infiltration localized within the deep white matter or peripheral to the lateral ventricles. Six nonoverlapping fields (×40 total magnification) were assessed for each section.

Statistical analyses

IBM SPSS for Windows version 23.0 (IBM Corporation, Armonk, NY) was used for statistical analyses. A chi-square test was used to test the differences of nominal values between 2 groups. All numerical data were tested for normality using Shapiro-Wilk tests. Extreme outliers were tested for exclusion with Smirnov-Grubbs tests. In the comparison of 2 groups, between-group differences in parametric data were tested for significance using *t*-tests, whereas Mann-Whitney *U* tests were used for nonparametric data. In the comparison of more than 2 groups, between-group differences in parametric data were tested for significance using one-way ANOVA, whereas Kruskal-Wallis tests were used for nonparametric data. Multiple post hoc comparisons were performed with Tukey or Dunnett tests. $P<.05$ was considered as statistically significant.

Results

Physiological variables

Eight of 10 fetuses in the EVE group and 6 of 8 fetuses in the LPS control group completed a predetermined 120-hour experimental period (Table 1). Key physiological data are presented in Figure 1. One experimental fetus (Figure 1, H) was excluded from general group comparison (Table 1) because of the presence of severe growth restriction (outlier; 494 g at 100-day GA). Another 2 fetuses were killed 24 hours and 36 hours after initiation of EVE therapy because of acute circuit failure (catheter flow was obstructed by intense fetal movement) and were excluded from further analyses.

Fetal BW and lung weight were significantly lower and CRL was significantly shorter in EVE group animals than in LPS control group animals. There were no significant differences in GA, sex ratio, weight-corrected CRL, weight-corrected lung weight, brain weight, and weight-corrected brain weight at the conclusion of the 120-hour study between the LPS control and EVE group animals over the experimental period (Table 1).

For EVE group animals, cord blood gas data had pO_2 values within the target range (15−30 mm Hg). pH, pCO_2, BE, SO_2, O_2, CtO_2, Na^+, K^+, Ca^{2+}, Cl^-, hemoglobin, lactate level, glucose, and activated clotting time also remained clinically acceptable and within an expected reference range (Table 2).

Statistically, AST, ALT, GGTP, GLDH, and BUN levels and the BUN to creatinine ratio were modestly but significantly higher in EVE group animals than those in the LPS control group animals, whereas no difference was found in total bilirubin, albumin, Mg, and *P* value. Creatinine level was significantly lower in EVE group than that in the control group at 120 hours (Table 3). Similar findings were seen in our previous work with this model, and the biological relevance of these modest changes remains unclear.

Plasma cortisol concentrations in EVE group animals were significantly increased over the 120-hour period of EVE therapy compared with values taken at the start of experiments (0 hour). Cortisol concentrations at the end of experiment in EVE group animals were significantly higher than those in the LPS control group animals (Figure 2, A). Plasma concentrations of ACTH in EVE group animals were significantly decreased over the 120-hour experimental period compared with values measured at 0 hour. ACTH concentrations taken at the end of experiments (120 hours) in EVE group animals were significantly lower than those in the LPS control group animals (Figure 2, B). In EVE group animals, plasma concentrations of IGF-1 were significantly decreased at 48, 72, 96, and 120 hours compared with measurements taken at 0 hour. IGF-1 concentrations at the end of experiment in EVE group animals were significantly lower than those in the LPS control group animals (Figure 2, C).

Ultrasound analysis for viability 48 hours after LPS injection confirmed fetal cardiac function in all LPS control and EVE group animals. For ultrasound assessments performed before euthanasia, there was no significant difference in tricuspid and mitral valve E:A ratio, right and left MPI, PLI, and corrected CO between the LPS control and EVE group animals. The direction of flow through the ductus arteriosus was from right to left in each of 8 EVE group animals (Table 4).

Individual case information (Figure 1, A−H) is as follows (Table 5):

Ex vivo uterine environment group animals 1 and 2 (cases A and B)

All measured physical variables remained within the respective reference ranges. Normal intermittent active fetal swallowing movements, breathing movements, gross fetal body movements, and flexure and extension of limbs were observed throughout the assessed period. There were no edema, ascites, pleural effusion, and bleeding. Brain injuries (hemorrhage and WMI) were not detected histopathologically. No bacteria were identified from blood and AF cultures.

Ex vivo uterine environment group animal 3 (case C)

Blood pressure and total circuit flow sharply decreased from 1 hour after induction of EVE therapy and was controlled by 3 doses of Dex (total=0.45 mg) in addition to the predetermined treatment (volume load and primary HC administration). All measured physical parameters were subsequently maintained within respective reference ranges for the first 60 hours. Normal intermittent active fetal swallowing movements, breathing movements, gross fetal body

TABLE 1
Comparison of fetal data at necropsy

Variable	LPS control group	EVE group	Statistical test	*P* value
Planned number	8	10		
Survival number	6	8		
Number for statistics	6	7		
Gestational age at LPS injection (d)	92.8±0.8	92.1±0.7		
Gestational age at induction of EVE therapy (d)	-	94.1±0.7		
Gestational age at conclusion (d)	99.8±0.8	99.1±0.7	*t*-test	.122
Sex (male/female)	0.5	3/4	Chi-square test	.383
Body weight (g)	1051 [973−1090]	869 [816−883][a]	Mann-Whitney *U* test	.035
Crown-rump length (cm)	32.1±1.0	30.3±1.2[a]	t-test	.013 [−3.14 to .46]
Weight-corrected crown-rump length (cm/kg)	31.1±1.8	34.5±4.5	*t*-test	118
Lung weight (g)	38.7±1.2	34.8±2.5[a]	*t*-test	.004 [−6.33 to 1.5]
Weight-corrected lung weight (g/kg)	37.6±2.7	39.6±5.7	*t*-test	.444
Brain weight (g)	26.5±1.6	25.1±1.0	*t*-test	.093
Weight-corrected brain weight (g)	25.1 [23.3−27.0]	28.7 [22.6− 30.0]	Mann-Whitney *U* test	.101

Respective values are expressed as group mean±SD and significant *P* values with 95% confidence interval were indicated for *t*-test. Respective values are expressed as mean [interquartile range] for Mann-Whitney *U* test. Significant difference vs value for the control group is indicated.

EVE, ex vivo uterine environment; *LPS*, lipopolysaccharide.

[a] $P<.05$.

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movements, and flexure and extension of limbs were observed in the first 60 hours. Circuit flow was interrupted for approximately 5 minutes with an unexpected occlusion of catheter flow at 60 hours after EVE therapy. Circuit flow was restored with changes in catheter position and angle. To prevent further deterioration in circuit performance, fetal movement was reduced through intravenous midazolam administration (0.15 mg/kg/h) until the end of the experimental period. There was no evidence of pleural effusion and bleeding, although there was slight edema and ascites. The review of H&E−stained coronal sections (mammillary bodies level) revealed the presence of focal coagulation necrosis and cellular infiltration consistent with periventricular leukomalacia. No bacteria were identified from blood and AF cultures.

Ex vivo uterine environment group animal 4 (case D)

Blood pressure and total circuit flow sharply decreased from 30 minutes after the induction of EVE therapy and was controlled by 3 doses of Dex administration (total=0.45 mg) in addition to the predetermined treatment. All measured physical variables subsequently remained within the desired reference ranges. Normal intermittent active fetal swallowing movements, breathing movements, gross fetal body movements, and flexure and extension of limbs were present throughout the assessed period. There were no edema, ascites, pleural effusion, and bleeding. The review of H&E−stained coronal sections (anterior basal ganglia level) revealed the presence of focal coagulation necrosis and cellular infiltration consistent with periventricular leukomalacia. No bacteria were identified from blood and AF cultures.

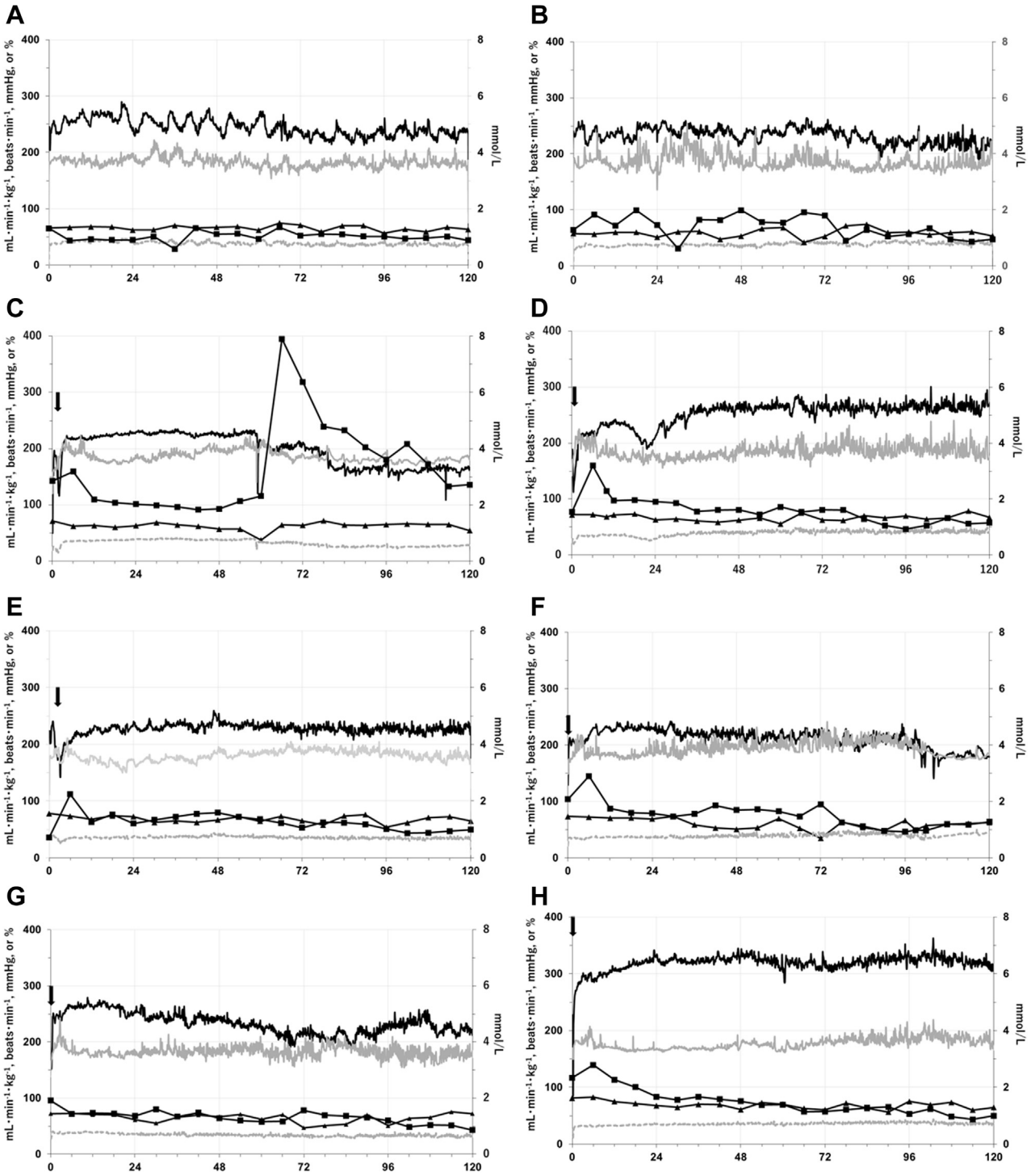
Ex vivo uterine environment group animal 5 (case E)

Blood pressure and total circuit flow sharply decreased from 1.5 hours after induction of EVE therapy and was controlled by 2 doses of Dex administration (total=0.3 mg) in addition to the predetermined treatment. All measured physical variables subsequently remained within the desired reference ranges. Normal intermittent active fetal swallowing movements, breathing movements, gross fetal body movements, and flexure and extension of limbs were present throughout the assessed period. There were no edema, ascites, pleural effusion, and bleeding. There were no histopathologic brain injuries. No bacteria were identified from blood and AF cultures.

Ex vivo uterine environment group animal 6 (case F)

Blood pressure and total circuit flow sharply decreased from 0.5 hours after induction of EVE therapy and was controlled by 2 doses of Dex administration (total=0.3 mg) in addition to the predetermined treatment. All measured physical parameters were subsequently maintained within the respective reference ranges in the first 108 hours. Normal intermittent active

FIGURE 1
Changes in fetal physiological and biochemical variables over time in the EVE group



The horizontal axis represents the time after induction of ex vivo uterine environment therapy (hours). The black solid lines show total oxygenator (circuit) blood flow (mL/kg/min); the gray solid lines show heart rate (beats/min); the gray dotted lines show mean arterial pressure (mm Hg); the black closed triangles show SO_2, arterial oxygen saturation (%); the black closed circles show pCO_2 (Torr); and the black closed squares show Lac, blood lactate level (mmol/L). Only the blood lactate levels use the right scale bar. The timing of refractory hypotension is also depicted (*black arrows*).

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fetal swallowing movements, breathing movements, gross fetal body movements, and flexure and extension of limbs were observed in the first 108 hours. After this time, the circuit flow started to fluctuate in response to fetal active movements and posture changes. To prevent further deterioration in circuit performance, fetal movement was reduced through intravenous midazolam administration (0.15 mg/kg/h) until the end of the experimental period. There were no ascites, pleural effusion, and bleeding, although there was slight edema. There were no histopathologic brain injuries. No bacteria were identified from blood and AF cultures.

Ex vivo uterine environment group animal 7 (case G)

Blood pressure and total circuit flow sharply decreased from 0.5 hours after induction of EVE therapy and was controlled by 2 doses of Dex administration (total=0.3 mg) in addition to the predetermined treatment. All measured physical variables subsequently remained within the respective reference ranges. Normal intermittent active fetal swallowing movements, breathing movements, gross fetal body movements, and flexure and extension of limbs were observed throughout the assessed period. There were no edema, ascites, pleural effusion, and bleeding. Brain injuries (hemorrhage and WMI) were not detected histopathologically. No bacteria were identified from blood although *Pseudomonas stutzeri* was identified in synthetic AF cultures taken both from the artificial uterus and the synthetic AF reservoir (before administration to the experimental system) at the conclusion of the experiment.

Ex vivo uterine environment group animal 8 (case H)

This fetus had growth restriction, with fetal BW (494 g), height (24 cm), and weight-corrected brain weight (45.3 g/kg) at the conclusion of the study. Blood pressure and total circuit flow sharply decreased from 0.5 hours after induction of EVE therapy and was controlled by 2 doses of Dex administration (total=0.6 mg) in addition to the predetermined treatment. All measured physical variables subsequently remained within the respective reference ranges. Normal intermittent active fetal swallowing movements, breathing movements, gross fetal body movements, and flexure and extension of limbs were observed throughout the assessed period. There were no edema, ascites, pleural effusion, and bleeding. The review of H&E–stained coronal sections (anterior basal ganglia level) revealed the presence of focal coagulation necrosis and cellular infiltration consistent with periventricular leukomalacia. No bacteria were identified from blood, although *Pseudomonas stutzeri* was identified in synthetic AF cultures taken both from the artificial uterus and the synthetic AF reservoir (before administration to the experimental system) at the conclusion of the experiment.

TABLE 2
Blood gas data throughout the experiments in the EVE group

Variable	Reference	EVE
pH	7.38±0.03	7.39±0.05
Base excess (mmol/L)	−0.2±1.8	0.8±3.6
pCO_2 (Torr)	42.4±2.9	43.6±3.8
pO_2 (Torr)	25.1±2.1	27.9±3.8
SO_2 (%)	65.5±5.9	64.4±7.9
CtO_2 (mmol/L)	8.9±1.2	9.2±1.3
Hemoglobin (g/L)	93±9	105±5
Glucose level (mmol/L)	1.6±0.2	3.2±1.3
Lactate level (mmol/L)	1.7±0.4	1.6±0.9
Na^+ (mmol/L)	134±3.1	143±5.7
K^+ (mmol/L)	4.0±0.3	3.5±0.5
Ca^{2+} (mmol/L)	1.4±0.1	1.3±0.1
Cl^- (mmol/L)	103±1.7	112±6.0
Activated clotting time (s)	—	203±24

Eight EVE group animals were analyzed throughout the experiment. Umbilical arterial blood was collected for blood gas data every 6 hours after the start of EVE therapy. Values are expressed as group mean±standard deviation.

The reference data were obtained from 13 age-matched (97±2 days gestational age), null-treatment fetuses, which were previously collected for our ovine databank.

Ca^{2+}, calcium ion; *Cl^-*, chloride ion; *CtO_2*, O_2 content (hemoglobin (g/dL) × 1.34 × SpO_2 (%)/100+ pO_2 × 0.003); *EVE*, ex vivo uterine environment; *K^+*, potassium ion; *Na^+*, sodium ion; *SO_2*, O_2 saturation.

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Ex vivo uterine environment group infection and inflammation

Although the plasma concentration of MCP-1 in LPS control and EVE group animals was significantly higher than that in the saline control group animals, no difference was found between EVE and LPS control group animals (Figure 3, A). Similarly, AF concentration of MCP-1 in LPS control and EVE group animals was significantly higher than that in the saline control group animals (Figure 3, B).

Evaluation of infection and inflammatory response at the end of experimental period (120 hours after ex vivo uterine environment or 168 hours after lipopolysaccharide injection for the control)

No bacteremia was found from blood cultures in any of the 8 EVE group animals, although *Pseudomonas stutzeri* was grown from the AF cultures in 2 EVE group animals (cases G and H) at the end

TABLE 3
Comparison of fetal chemical data at 120 hours

Variable	LPS control group	EVE group	Statistical test	*P* value
AST (U/L)	24 [22−28]	56 [40−138][a]	Mann-Whitney *U* test	.003
ALT (U/L)	3.0 [2.2−3.0]	9.5 [6.8−5.0][a]	Mann-Whitney *U* test	.008
GGTP (U/L)	22±9	48±16[a]	*t*-test	.012 [7−45]
GLDH (U/L)	10 [5−17]	17 [40−138] [a]	Mann-Whitney *U* test	.012
Total bilirubin (mg/dL)	1.9±0.5	2.4±1.9	*t*-test	.105
Albumin (g/dL)	1.9±0.1	2.0±0.2	*t*-test	.247
BUN (mg/dL)	13.5±3.2	20.3±6.5[a]	*t*-test	.03 [.8−12.8]
Creatinine (mg/d/L)	0.87±0.14	0.53±0.17[a]	*t*-test	.001 [−0.53 to −.16]
BUN/Cre ratio	15.7±4.6	38.0±4.1[a]	*t*-test	.001 [17.9−29.9]
Mg (mmol/L)	1.2±0.2	1.1±0.1	*t*-test	.397
P (mmol/L)	2.2±0.3	2.0±0.2	*t*-test	.07

Six LPS control and 8 EVE group animals were analyzed. Respective parametric values are expressed as group mean±standard deviation, and significant *P* values with 95% confidence interval were indicated for *t*-test. Respective nonparametric values are expressed as mean [interquartile range] for Mann-Whitney *U* test. Significant difference vs value for the control group is indicated.

ALT, alanine aminotransferase, *AST*, aspartate aminotransferase; *BUN*, blood urea nitrogen; *EVE*, ex vivo uterine environment; *GGTP*, gamma-glutamyl transpeptidase; *GLDH*, glutamate dehydrogenase; *LPS*, lipopolysaccharide; *Mg*, magnesium; *P*, phosphate.

[a] $P<.05$.

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of the experimental period (120 hours). There was no significant difference in plasma concentrations of MCP-1 between saline negative control, LPS control, and EVE group animals (one-way ANOVA; $P=.372$), and plasma TNF-α was not detected in any of the groups (data not shown).

The number of circulating WBCs in LPS control and EVE group animals was significantly higher than that in saline negative control group animals, whereas no difference was found between LPS control and EVE group animals. Similarly, the number of neutrophils in the LPS control and EVE group animals was significantly higher than that in saline negative control group animals; there was no difference between LPS control and EVE group animals. There was no difference in the number of lymphocytes between any of the groups. The number of monocytes was higher in LPS control group animals than that in saline negative control and EVE group animals (Figure 4, A).

For lung quantitative PCR, no significant differences in the relative mRNA expression of IL-1β, IL-6, TNF-α were identified between saline negative control, LPS control, and EVE group animals. The relative mRNA expression of MCP-1 in LPS control group animals was significantly higher than that in saline negative control and EVE group animals (Figure 4, B).

Brain histopathology

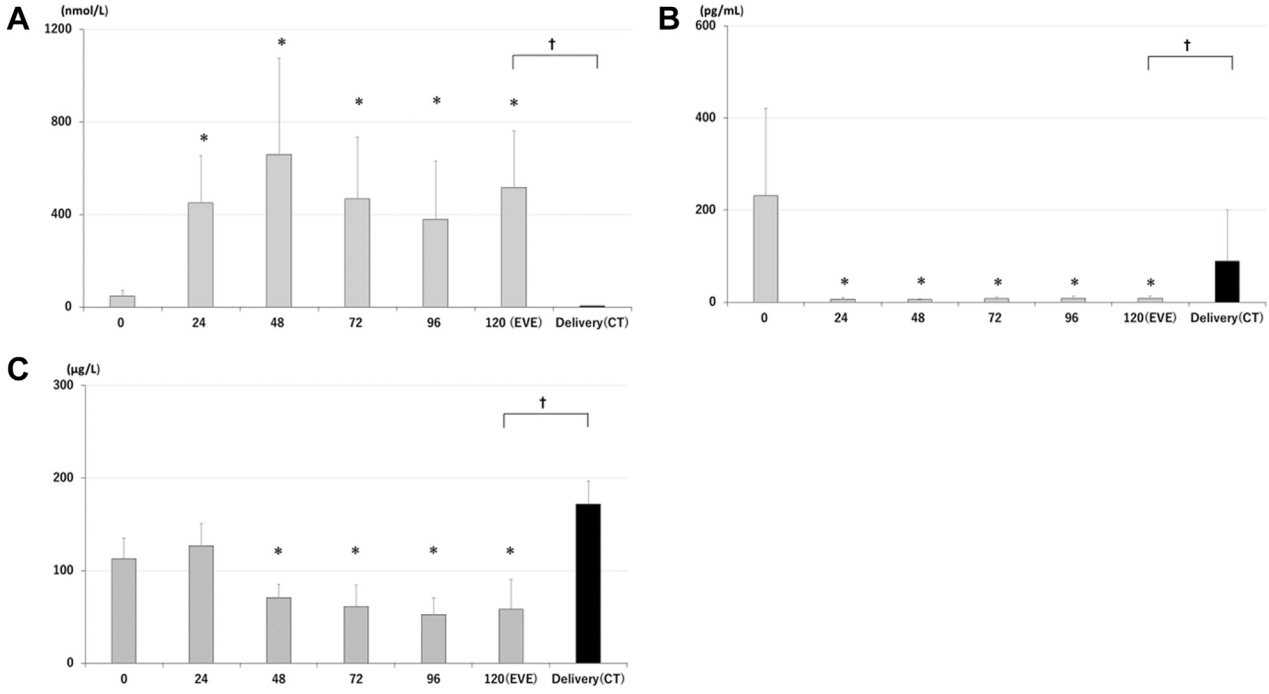
Three LPS control and 3 EVE group animals had WMI in H&E−stained coronal sections (anterior basal ganglia level or mammillary bodies level), whereas no WMI was found in any of the 9 saline negative control group animals. There was no evidence of hemorrhage or WMI in any of the remaining LPS control, EVE, or saline negative control group animals in gross anatomic and H&E−stained histopathologic analyses of coronal brain sections (Figure 5).

Comment

The primary finding of this study is that EVE tallowed a 120-hour period of survival in a group of 8 extremely preterm lambs compromised by a 48-hour antenatal exposure to intrauterine inflammation (Figures 1 and 3; Table 6). To our knowledge, this is the first report of an artificial placenta−based life support system being used to sustain extremely preterm fetuses (400−700 g), compromised by exposure to intrauterine inflammation—a common antecedent of extremely preterm delivery. Fetal growth restriction was observed in EVE group animals compared with LPS positive control (in utero) group animals, although EVE group animals were otherwise stable from a hemodynamic viewpoint after acute intensive management (Figure 1, Tables 1−3 and 5) and did not present evidence of altered cardiac parameters by ultrasound (Table 4). This is a particularly important finding as, ideally, fetal growth should be maintained as close to normal as possible in an artificial placenta platform. Given the differences in somatic growth and hormone concentrations identified in the 3 study groups, it appears that the observed growth restriction derives from an as-yet unidentified deficiency in our EVE therapy system, rather than LPS exposure itself.

All animals were free of bacteremia, and no additional systemic inflammatory changes were detected at the conclusion of treatment in the EVE group animals compared with LPS

FIGURE 2
Changes in the concentrations of cortisol, adrenocorticotropic hormone, and insulin-like growth factor 1 over time in the EVE group and comparisons between EVE and control groups at the end of the study



Six lipopolysaccharide positive control animals and 8 EVE group animals were analyzed for plasma concentrations of cortisol (panel A), adrenocorticotropic hormone (panel B), and insulin-like growth factor 1 (panel C). The horizontal axis represents the time after the induction of ex vivo uterine environment therapy (hours). All values are presented as bar charts with group mean and with whiskers representing standard deviation. Gray bar indicates EVE group animals. Black bar indicates control animals. Differences of values over time in EVE group animals were tested for significance using repeated one-way analysis of variance. Multiple post hoc comparisons were performed using Dunnett test compared with the value at 0 hour; The * symbol indicates $P<.05$ accepted as significant. Differences of values at the end of the experimental period between EVE and control group animals were tested using *t*-test; The †symbol indicates $P<.05$ accepted as significant.

CT, control; *EVE*, ex vivo uterine environment.

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control group animals (Figures 3 and 4; Tables 5 and 6). Although a treatment time of 120 hours may be limited in terms of clinical use, data from this study show the potential of EVE therapy as a clinical platform for extremely preterm infants exposed to intrauterine inflammation.

Maintenance of key physiological parameters

Key physiological parameters and blood lactate levels generally remained within their reference ranges, or rapidly returned to reference range after EVE therapy was started and/or refractory hypotension was successfully treated (Figure 1; Table 2). Total lung flow volume was maintained within normal range (150−300 mL/kg/min), which was similar to that shown by our previous study using extremely preterm lamb model.[8] Although fetal hypoxia ($pO_2<20$ Torr) is likely to be induced by LPS and reported to become a factor of brain injury,[40,41] pO_2 and CtO_2 were maintained within a reference range at least after the induction of EVE therapy (Table 2). However, refractory hypotension and the subsequent transient elevation of lactate level might reflect fetal ischemic changes (Figure 1).

Ultrasound data

The main aim of these ultrasound measurements was to determine cardiac dysfunction in EVE therapy animals, and the reference range was described in a previous report using extra preterm lamb model.[8] Differences in any items between LPS control and EVE group animals were not demonstrated, and the values of all items in both group animals were within the reference range. Fetal circulation (R→L direction through the ductus arteriosus) was maintained with EVE therapy over the 120-hour experimental period.

Infection and inflammation

All animals were free of bacteremia throughout the experiment. In terms of inflammation at 48 hours after IA LPS injection, MCP-1 has been reported to be significantly increased both in plasma and AF for extremely preterm fetuses.[25,26] MCP-1 was evaluated as an indicator of inflammation. EVE group animals had a significant increase in MCP-1, which was equivalent to that of

TABLE 4
Comparison of fetal cardiac ultrasound data at study conclusion

Variable	LPS control group	EVE group	*P* value
Tricuspid valve E:A ratio	0.72±0.08	0.68±0.08	.414
Mitral valve E:A ratio	0.68±0.11	0.66±0.08	.774
Right Myocardial Performance Index	0.43±0.04	0.43±0.08	.417
Left Myocardial Performance Index	0.41±0.03	0.42±0.03	.99
Preload index	0.35±0.12	0.38±0.14	.67
Corrected cardiac output (mL/min/kg)	530±60	511±122	.74
Direction of ductus arteriosus flow	Right → left	Right → left	

Six LPS control and 8 EVE group animals were analyzed. Respective values are expressed as group mean±standard deviation. *t*-test was conducted for statistical analysis. $P<.05$ was considered as significant difference. Blood flow from the pulmonary artery to the descending aorta was determined as right to left directional flow.

EVE, ex vivo uterine environment; *LPS*, lipopolysaccharide.

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LPS positive control group animals (Figure 3, A and B). Thus, intrauterine inflammation was induced by IA LPS injection, and EVE surgery and platform maintenance could be conducted using extremely preterm lambs compromised by intrauterine inflammation.

WBC and neutrophil levels increased both in LPS control and EVE group animals and were equivalent at the end of the 120-hour experimental period (Figure 4, A). Reportedly, in preterm sheep, peripheral WBC and neutrophil levels increased over 4−7 days after IA LPS exposure. WBC counts then returned to control levels by 15 days, whereas neutrophils counts remained increased at 14 days after LPS exposure.[42−44] Thus, this increase of WBCs and neutrophils seemed not to be induced by additional infection but by the IA LPS injection.

MCP-1 transcript expression in lung tissues of LPS control group animals was significantly higher than that of saline negative control and EVE group animals at the conclusion of the experiment, whereas all other measured transcripts returned to baseline (Figure 4, B). In preterm fetal sheep, IA LPS injection induces large increases of proinflammatory cytokine responses in the fetal lung.[25,26] Those inflammatory responses (IL-1β, IL-6, TNF-α) are reported to return to baseline by 7−15 days after IA injection,[44] and our data suggest that the adaptation of compromised extremely preterm fetuses to EVE therapy does not exacerbate existing inflammation or gross brain injury.

Success rate

The survival rate with EVE therapy was 80%, whereas that with the control animals was 75% (Table 6). Two EVE animals died because of catheter-based circuit compromise. Two control animals also died owing to unidentified reasons. Reportedly, intravenous LPS injection causes fetal systemic inflammation, thereby leading to fetal death.[45] However, fetal death due to IA LPS injection has not been reported in extremely early GA experiments, although this may be due to more abbreviated study periods being reported.[25,26,46] In this study, no fetal death was detected by ultrasound in either the LPS positive control or EVE group animals during the first 48 hours after IA LPS injection.

Limitations

The primary limitation of this study is the small sample size and short trial period. As previously described, a 5-day maintenance period was selected based on several considerations. First, balancing the substantial amount of infrastructure and constant monitoring limits the sample number achievable.[8] Second, as this was the first attempt to adapt extremely preterm fetuses compromised by intrauterine inflammation to an artificial placenta system, we elected to limit the duration of the experiment to 5 days, allowing us to establish the acute efficacy of our system.[8]

Although we assessed a range of key physiological and hematological variables in determining fetal well-being over the 120-hour experiments, we did not perform comprehensive assessments of organ development. Given the nature of the experimental design, it was also not possible to assess the hemodynamic impact of IA LPS on LPS control group animals without incorporating additional surgical interventions. We elected not to pursue this approach in this study because we wished to focus on the comparison of fetal development in the absence of potential confounding from fetal surgery necessary to introduce catheters and monitoring probes; future studies may require the addition of an instrumented LPS control group along with a noninstrumented group.

The use of glucocorticoids appears to be essential for current EVE therapy in the maintenance of extremely preterm lambs at the border of viability, although it is also well known that the use of glucocorticoids to premature fetuses affects maturation of vital organs such as the brain and lungs, inflammatory markers, and death.[47−50] It is also worth noting that glucocorticoids are also effective anti-inflammatory agents, which should be taken into account when assessing the inflammatory data

TABLE 5
Case summary of EVE group animals

EVE animals	EVE 1 (A)	EVE 2 (B)	EVE 3 (C)	EVE 4 (D)	EVE5 (E)	EVE 6 (F)	EVE 7 (G)	EVE 8 (H)
Swallowing movement	+	+	(24–62 h)+	+	+	(24–108 h)+	+	+
Breathing movement	+	+	(24–62 h)+	+	+	(24–108 h)+	+	+
Gross body movements	+	+	(24–62 h)+	+	+	(24–108 h)+	+	+
Flexure and extension of limbs	+	+	(24–62 h)+	+	+	(24–108 h)+	+	+
Edema	-	-	+	-	-	+	-	-
Ascites	-	-	+	-	-	-	-	-
Pleural effusions	-	-	-	-	-	-	-	-
Total dose of dexamethasone administration to control refractory hypotension (mg)	-	-	0.45	0.45	0.3	0.3	0.3	0.6
Brain bleeding	-	-	-	-	-		-	-
White matter injury	-	-	+	+	-	-	-	+
Blood culture	-	-	-	-	-	-	-	-
Culture from AF in artificial womb	-	-	-	-	-	-	*Pseudomonas stutzeri*	*Pseudomonas stutzeri*
AF culture	-	-	-	-	-	-	*Pseudomonas stutzeri*	*Pseudomonas stutzeri*
Specified issue	-	-	Circuit trouble owing to a kink at the tip of the catheters	-	-	Circuit trouble owing to a kink at the tip of the catheters		Severe fetal growth restriction (weight=494 g, height=24 cm, weight-corrected brain weight=45.3 g/kg at conclusion)

The + symbol indicates present and — indicates absent.

Normal intermittent active fetal swallowing movement, breathing movements, gross fetal body movements, and flexure and extension of limbs were assessed at least every 6 hours. Edema, ascites, pleural effusion, bleeding, and another specific issue were identified during necropsy after 120 hours of EVE therapy. White matter injury was identified in hematoxylin and eosin–stained coronal sections. Samples for culture bottles were collected from fetal umbilical artery, synthetic AF in artificial womb, and sterilized tubes at the end of the experimental period.

AF, amniotic fluid; *EVE*, ex vivo uterine environment.

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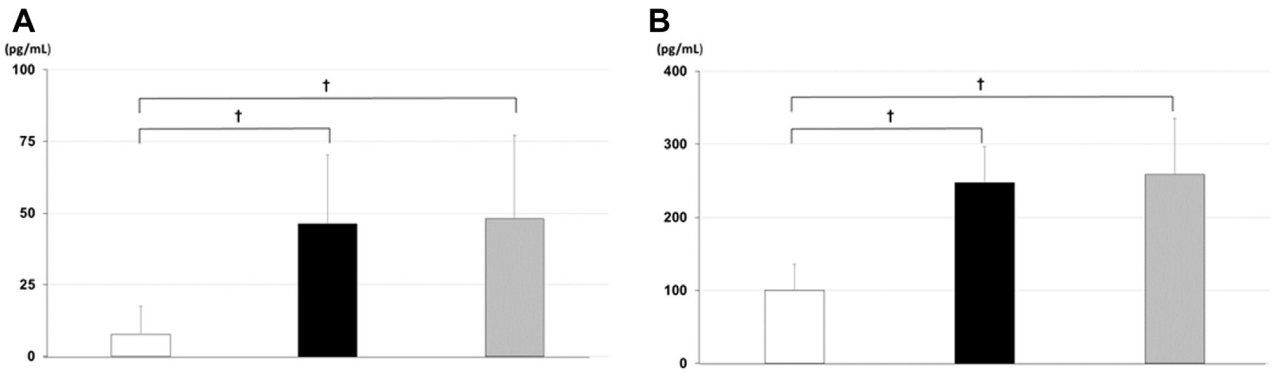
FIGURE 3
Comparison of concentration values of monocyte chemoattractant protein 1 in fetal plasma and amniotic fluid at 48 hours after lipopolysaccharide (LPS) injection



Nine saline negative control (saline treatment was conducted at a gestational age of 93 days), 9 LPS control (LPS treatment was conducted at a gestational age of 93 days), and 8 ex vivo uterine environment (EVE) group animals (LPS treatment was conducted at a gestational age of 93 days) were analyzed. Panel A shows the comparison of fetal plasma concentration and panel B shows the comparison of amniotic fluid concentration of monocyte chemoattractant protein 1. All values are presented as bar charts with group mean and with whiskers representing standard deviation. The white bars indicate negative control group. The black bars indicate the positive control group. The gray bars indicate the EVE group. Differences of values between the groups were tested for significance using one-way analysis of variance. Multiple post hoc comparisons were performed using Turkey test. The † symbol indicates $P<.05$ accepted as significant.

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presented in this study. This is also important as the LPS control group animals were not exposed to steroids or antibiotics, both of which have the potential to modulate inflammatory and injury responses. Given the potential for glucocorticoid-induced changes to fetal development, extensive assessments of optimal dosing and the impact of glucocorticoids on inflammation and growth will be key elements of future studies; such analyses will be facilitated by long-term studies using our refined EVE therapy system and will be essential to demonstrate the clinical utility of this concept for the management and support of extremely preterm infants.

An additional limitation of importance is the inflammatory stimuli employed. This study used LPS, which is a sterile modulator of toll-like receptor 4 signaling. It has the advantage of delivering a standardized, albeit self-limited, proinflammatory response. However, it neither faithfully replicates the polyvalent stimulation derived from an active microbial or fungal infection of the uterus nor facilitates fetal sepsis, which is similarly associated with extremely preterm delivery.[51,52] Delivery timing was set at 48 hours after IA LPS injection based on previous studies.[25,26,43,44] Although this setting would enable us to evaluate if the induction of EVE therapy in extremely preterm lambs exposed to intrauterine inflammation was feasible, it might not be appropriate to evaluate the resolution of fetal inflammations because many inflammatory responses induced by IA LPS return to the standard level in 7 days.[43,44]

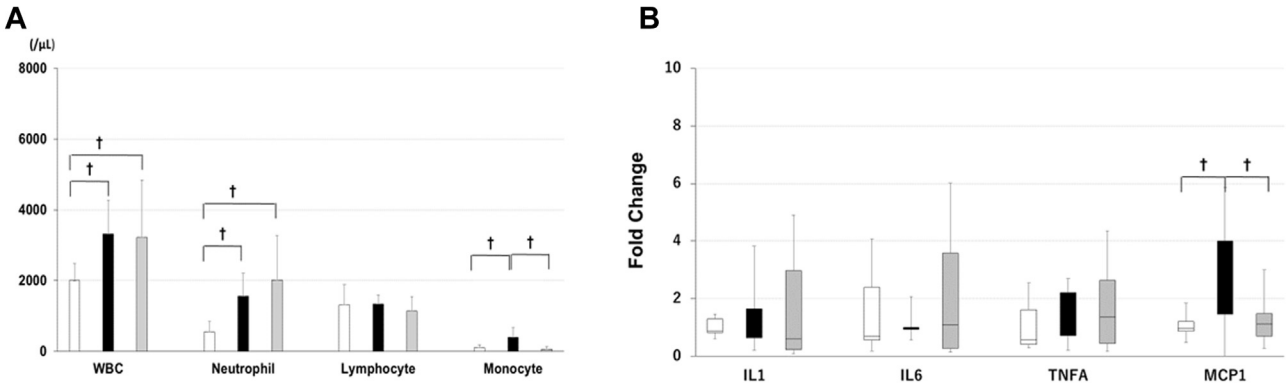
Challenges yet to be overcome for clinical application

Refractory hypotension

In this study, HC was administered to manage refractory hypotension after the induction of EVE therapy.[8] However, hypotension (which could be managed comparatively easily in previous studies using LPS-naive fetuses) could not be easily controlled even with the use of additional volume expansion and HC administration in the majority of EVE group animals exposed to intrauterine inflammation (EVE 1−8). Profound refractory hypotension in a number of these animals occurred within the first 3 hours after the induction of EVE therapy. Although total lung flow and blood pressure were initially maintained within the reference range after adaptation to EVE therapy, both metrics started to degrade shortly thereafter.

Hypotension is frequently identified in preterm infants, and its incidence is inversely related to GA at delivery likely because of a host of factors such as immature myocardium, immature autonomic nervous system, and relative adrenal insufficiency.[53−60] Approximately 25% of ELBW infants with hypotension do not respond to either volume expansion or inotropic drug and are treated by HC, independent of serum cortisol concentrations, to normalize blood pressure.[61−63] In our study, HC treatment and volume expansion were insufficient for a number of EVE group animals exposed to intrauterine inflammation to recover from refractory hypotension. Although the mechanism for this is unclear, it may be because chorioamnionitis also releases inflammatory mediators that increase vascular permeability and vasodilation and in doing so predispose the fetus to refractory

FIGURE 4
Differential cell counts (blood) and cytokine mRNA expression (lung) at the end of the experiment



Nine saline negative (saline treatment; data taken from our ovine bank age-matched at 99.9±0.7 days of gestational age), 6 lipopolysaccharide (LPS) control, and 8 ex vivo uterine environment (EVE) group animals were analyzed. Panel A, white blood corpuscles. All values are presented as bar charts with group mean and with whiskers representing SD. The white bars indicate the saline negative control group. The black bars indicate the LPS control group. The gray bars indicate the EVE group. Differences of values between the groups were tested for significance using one-way analysis of variance. Multiple post hoc comparisons were performed using Turkey test. The † symbol indicates $P<.05$ accepted as significant. In panel B, tumor necrosis factor α and monocyte chemoattractant protein 1. Relative fold changes in cytokine (interleukin-1β, interleukin-6, tumor necrosis factor α, and monocyte chemoattractant protein 1) mRNA expression in lung tissue samples. All values are presented as box plots with group median and with whiskers representing maximum and minimum values. White box indicates saline negative control group and black box indicates LPS control group animals. Gray box indicates EVE group animals. Respective differences of values were tested for significance using Kruskal-Wallis test. Multiple post hoc comparisons were performed using Turkey test. The † symbol indicates $P<.05$ accepted as significant.

IL, interleukin; *MCP-1*, monocyte chemoattractant protein 1; *TNF-α*, tumor necrosis factor α; *WBC*, white blood corpuscle.

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hypovolemia or hypotension.[63] Administration of the strong glucocorticoid Dex might improve the clinical picture by compensating for relative steroid insufficiency and contributing to reduced inflammation through its anti-inflammatory activity. Given the time scale involved, it is also possible that several hours are required for the benefit of glucocorticoid signaling changes to translate into improvements in physiological parameters and that additional steroid administration (including Dex) is unnecessarily excessive.

Although the optimal dose and timing remains unclear, HC and Dex coadministration assisted us in achieving the 120-hour survival time for 8 of 10 EVE group animals exposed to intrauterine inflammation. However, EVE group animals still had transient increases in lactate level after recovery from hypotension. HC and Dex therapy resulted in high concentrations of fetal cortisol and may have suppressed ACTH secretion (Figure 2, A and B). Thus, optimization of appropriate timing and dose and evaluation of long-term effects of glucocorticoid treatment will become key elements of future work to minimize adverse effect to growth or neurodevelopment.

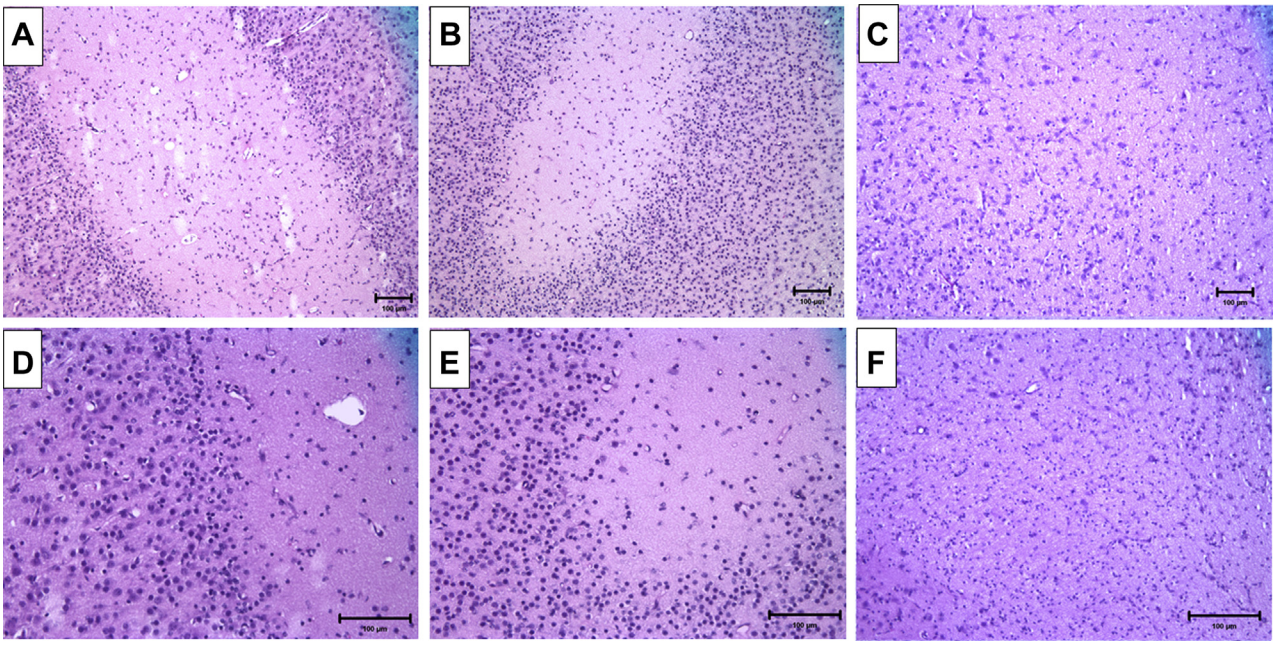
Growth restriction

EVE group animals had growth restriction compared with LPS control animals. LPS control group animals had ostensibly normal growth[8] despite being subjected to the same IA LPS exposure as growth-restricted EVE group animals. These data suggest that EVE group animals exposed to intrauterine inflammation are susceptible to extrauterine growth restriction (EUGR), the most frequent morbidity among premature babies especially those weighing <1500 g under current neonatal clinical settings.[64,65]

The primary cause of EUGR is widely considered to be nutrient insufficiency.[66,67] Although EVE group animals were given the same nutrient supplementation,[8] that level of support may not meet the requirements of extremely preterm lambs exposed to intrauterine inflammation. The metabolic response to and nutritional requirements of sepsis in the neonate are not well defined; septic neonates are reported to require higher-energy delivery during the acute phase of their illness. In adults, sepsis alters protein requirements more acutely by its effect on cytokine-mediated muscle catabolism. A similar requirement may be observed in extremely preterm fetuses. No study has assessed whether provision of extra protein will reduce this catabolic response for neonates, but at this point, it seems prudent to provide at least 2.5 g/kg per day of protein to the septic infant.[68,69] Thus, optimization of appropriate nutrition for extremely preterm infants with intrauterine inflammation may be key for future treatment.

HC and Dex use also might be a cause of growth restriction. The use of corticosteroids such as HC and Dex has been established to prevent or reduce chronic lung injury for premature babies. However, neonatal Dex has also been associated with somatic growth impairment, both during treatment ("early growth

FIGURE 5
Representative images of brain histology



Six lipopolysaccharide (LPS) control and 8 ex vivo uterine environment (EVE) group animals (EVE 1—8) were analyzed with hematoxylin and eosin (H&E) stain. Six fields from four 5-mm serial sections were assessed for each animal. Three control and 3 EVE group animals (EVE 3, 4, and 8) had white matter injury (WMI). Representative image of WMI from 1 of injured LPS positive control group animals (panels A and D) and EVE 4 in EVE group animals (panels B and E) and representative image of non-WMI from saline negative control group animals (panels C and F) identified in investigation with H&E staining showing necrosis and cellular infiltration. Images are inspected at ×100 total magnification (panels A—C) and ×200 (panels C—F); scale bar represents 100 μm.

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restriction")[70,71] and at follow-up of previously Dex-treated newborns at ages ranging between 2 and 17 years,[72—75] whereas no significant effect of postnatal HC therapy has been found on somatic growth at ages ranging between 2 and 17 years.[76—79] Although it is presently unclear which impact the combined effects of preterm birth and corticosteroid-related early growth retardation may have on subsequent growth, whether glucocorticoid-treated babies sometimes demonstrate catch-up growth is presumed to underlie long-term health risks.[80] Appropriate

TABLE 6
Success rate over time in the EVE group

Variable	LPS control group	EVE group
Total number	8	10
Survival number	6	8
Survival rate (%)	75	80
Completion number without bacteremia among surviving animals	-	8
Completion rate without bacteremia among surviving animals (%)	-	100
Completion number without brain injury among surviving animals	3	5
Completion rate without brain injury among surviving animals (%)	50.0	62.5

Bacteremia was determined by microbial culture. Brain injury was determined by the presence of intraventricular hemorrhage, white matter injury identified in gross anatomic observation, and historical assessment of the brain sections.

EVE, ex vivo uterine environment; *LPS*, lipopolysaccharide.

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use of glucocorticoids will be key for future studies in this area (Figure 2, A and B).

IGF-1 values in EVE group animals were decreased over the experiment and were significantly lower than that in the LPS control group animals at the conclusion of the experiment (Figure 2, C). Whereas IGF-2 is the primary growth factor underpinning embryonic growth, the dominant fetal growth regulator is IGF-1, produced mainly by the fetal liver in addition to AF and other organs.[81−83] Umbilical cord IGF-I levels correlate closely with birthweight and there has been a report of 1 human patient with an IGF-1 gene deletion who presented with severe IUGR.[84,85] Similarly, direct fetal infusion of IGF-1 promotes fetal substrate uptake and inhibits fetal catabolism.[86] In contrast to the postnatal situation, the dominant driver of circulating fetal IGF-1 regulator is not only the growth hormone (GH)[87−89] but also fetal insulin, which in turn is predominantly regulated by fetal glucose availability.[90,91] Thus, in this study, inappropriate nutrition (inappropriate glucose or insulin level), excessive corticosteroid dose (especially Dex) that might interfere with GH pulsation and decrease total GH secretion and IGF-1,[92,93] and the absence of AF-derived IGF-1 may combine to result in an overall deficiency of IGF-1 levels and become a factor in growth restriction. Therefore, monitoring IGF-1 and IGF binding proteins[94] as an indicator of fetal growth in the management of nutrition and glucocorticoid use or replenishing IGF-1 may improve outcomes in this experimental system.

Brain injury

Gross anatomic observations and assessment of H&E−stained brain sections identified the presence of pathologic lesions in 3 of the LPS control group animals and 3 of 8 EVE group animals (EVE 3, 4, and 8).

There was no evidence of hemorrhage in any of the 6 LPS control and 8 EVE group animals or of WMI in the remaining 3 control and 6 EVE animals in gross anatomic and H&E−stained histopathologic analyses of coronal brain sections (Table 5; Figure 5).

In addition, WMI has been associated with prematurity especially below 32 weeks' gestation, typically localized to the periventricular white matter area in a diffuse or focal pattern.[95,96] In addition, inflammation during fetal and neonatal development alone has been linked to brain injury.[23,97,98] Similarly, extremely preterm fetal sheep (GA of 91−93 days) exposed to LPS reported WMI.[41,99] Although the cause of this injury in this study is unclear, it might derive from hypoxic-ischemic change or proinflammatory cytokines mediating direct effects.[8,30,100−103]

Conclusion

Extremely preterm sheep fetuses compromised by a 48-hour antenatal exposure to intrauterine inflammation were maintained for a period of 120 hours using an artificial placenta−based life support platform. These studies have identified 4 key issues for the future development of this technology for clinical application: (1) refractory hypotension, (2) growth restriction, (3) brain injury, and (4) stabilizing circuit performance to protect against WMI.

As such challenges are largely absent in studies based on healthy sheep fetuses, additional experiments of this nature are essential for further development of this technology. Although still preliminary, these novel findings using extremely preterm lambs compromised by exposure to intrauterine inflammation do demonstrate that, with significant additional development, there is potential clinical utility for artificial placenta technology to support extremely preterm infants. ■

GLOSSARY

Artificial placenta: a gas exchange device comprising 1 or more membranous oxygenators connected to the umbilical vessels by indwelling catheters. Circuit pressure is provided by the fetal heart.

Ex vivo uterine environment (EVE) therapy: the use of an artificial placenta to provide life support to an extremely preterm infant by simulating placental gas exchange.

Extremely preterm infants: infants born close to the border of viability, that is, 21−24 weeks' gestation.

Glucocorticoids: synthetic steroid hormones that exert pleiotropic signaling activity through glucocorticoid receptor activation. Widely used in obstetrics to precociously mature the preterm lung in anticipation of preterm delivery.

Lipopolysaccharides: large lipid and polysaccharide molecules found in the outer layer of Gram-negative bacteria. Lipopolysaccharides elicit a robust inflammatory response that can be used to simulate intrauterine inflammation and generate chorioamnionitis.

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